

Studies of intestinal morphology and cathepsin B expression in a transgenic mouse aiming at intestine-specific expression of Cath B-EGFP

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Abstract

Cathepsin B has been shown to not only reside within endo-lysosomes of intestinal epithelial cells, but it was also secreted into the extracellular space of intestinal mucosa in physiological and pathological conditions. In an effort to further investigate the function of this protease in the intestine, we generated a transgenic mouse model that would enable us to visualize the localization of cathepsin B *in vivo*. Previously we showed that the A33-antigen promoter could be successfully used *in vitro* in order to express cathepsin B-green fluorescent protein chimeras in cells that co-expressed the intestine-specific transcription factor Cdx1. In this study an analog approach was used to express chimeric cathepsin B specifically in the intestine of transgenic animals. No overt phenotype was observed for the transgenic mice that reproduced normally. Biochemical and morphological studies confirmed that the overall intestinal phenotype including the structure and polarity of this tissue as well as cell numbers and differentiation states were not altered in the A33-CathB-EGFP mice when compared to wild type animals. However, transgenic expression of chimeric cathepsin B could not be visualized because it was not translated *in situ* although the transgene was maintained over several generations.

Keywords: A33-antigen promoter; cysteine cathepsins; enhanced green fluorescent protein; intestine.

Introduction

In the gastrointestinal tract, like in any other tissue, a complex proteolytic network is responsible for controlling and

regulating tissue homeostasis. Cathepsins are part of this network and are classified as aspartic, cysteine, and serine proteases. Elucidating the roles of these enzymes is of crucial importance since cathepsins are known to exhibit diverse functions in human physiology but also under pathological conditions, such as cancer (Riese and Chapman, 2000; Turk et al., 2001; Friedrichs et al., 2003; Buth et al., 2007; Vasiljeva et al., 2007; Brix et al., 2008). There are 11 cysteine cathepsins known to be encoded by the human genome whereas rodents express a larger group of these enzymes due to additional cysteine cathepsin family members that are placenta-specific and not found in other species (Deussing et al., 2002; Sol-Church et al., 2002; Mason, 2008). Some of these proteolytic enzymes, such as cathepsin B, are ubiquitously expressed, whereas others are considered to be cell- or tissue-specific in their tasks (Brix et al., 2008; Reiser et al., 2010; Dauth et al., 2011). Because of their functional diversity, cysteine cathepsins have been viewed as promising targets for the treatment of various disorders, including neurodegenerative diseases, osteoporosis and a variety of malignancies (Mohamed and Sloane, 2006; Turk, 2006; Bromme and Lecaille, 2009; Arampatzidou et al., 2011). Among them, cathepsin B gained significant attention since it is not only an endopeptidase, like most of the cysteine cathepsins, but it can also act as an exopeptidase (Musil et al., 1991; Mort and Buttle, 1997; Nagler et al., 1997; Kos et al., 2005; Brix et al., 2008). In the intestine, cathepsin B has been proposed to have a direct role in collagen degradation for extracellular matrix (ECM) remodeling (Vreemann et al., 2009), while it has also been identified as an important player during inflammatory bowel disease (Menzel et al., 2006). In intestinal epithelial cells, cathepsin B has been predominantly localized within endo-lysosomes (Mayer et al., 2006; Arampatzidou et al., 2011), whereas other cysteine proteases such as cathepsins K and L were found in association with the apical plasma membrane (Mayer et al., 2006) and affected the structural integrity of the intestine by interfering with the constitution of the basal lamina (Dauth et al., 2011). These observations suggested different trafficking routes for the various cathepsins within the cells of the intestinal mucosa that could also imply different functions for each of these enzymes.

Therefore, we wanted to study the transport pathway of cathepsin B by using enhanced green fluorescent protein (EGFP) tagging in an intestine-specific setting. For this purpose, we chose the A33-antigen promoter since the expression of genes under control of this promoter depends on the presence of the intestine-specific transcription factor Cdx1 (Johnstone et al., 2002). The A33 antigen is a transmembrane

protein that is expressed only in the intestine and is specifically localized at the basolateral membrane of intestinal epithelial cells of all lineages (Johnstone et al., 2000). The A33-antigen promoter contains positive *cis*-regulatory elements, including caudal-related homeobox (Cdx1) binding sites. Cdx1 is an intestine-specific transcription factor which is crucial for A33-antigen promoter activation and could thereby induce expression of the chimeric protein cathepsin B-EGFP (Johnstone et al., 2000). By proof-of-principle studies we confirmed that the A33-antigen promoter can serve as a tool for induction of Cdx1-dependent CathB-EGFP expression *in vitro* (Mayer et al., 2008). In the present study, we used a similar approach for expressing CathB-EGFP in the intestine of a transgenic mouse model that was expected to enable us to elucidate and directly visualize the function and trafficking of cathepsin B *in vivo*.

Results

Generation of transgenic mice expressing cathepsin B-EGFP under the control of the A33-antigen promoter

A transgenic mouse model was established in order to be able to study the trafficking and function of cathepsin B *in situ*. These transgenic animals were expected to express the chimeric protein cathepsin B-EGFP under the control of the A33-antigen promoter, using the principle of intestine-specific expression of the A33 antigen (Mayer et al., 2008). C57BL/6NCrl mice were used for this study and the linearized A33-CathB-EGFP construct was microinjected into the male pronucleus of fertilized oocytes. After integration of transgene was proven to be successful, one transgenic founder was used for generation of transgenic offspring which were comparable to wild type littermate controls with respect to weight, size and breeding behavior. In addition, A33-

CathB-EGFP transgenic mice appeared at the expected Mendelian frequency. Tail biopsies of the transgenic animals were analyzed in order to examine the incorporation of the transgene by using semi-quantitative polymerase chain reaction (PCR) analysis. Different amounts of transgene were detected in the A33-CathB-EGFP mice of different generations (Figure 1A) but the transgene was kept over the three generations analyzed (Figure 1B).

Expression of A33-CathB-EGFP transgene

The next step was to check the expression of the A33-CathB-EGFP transgene for presence in the intestine of transgenic mice. For this purpose, intestinal tissue was obtained from both transgenic and wild type mice and was divided into duodenum, jejunum, ileum, and colon. Liver was also obtained since it served as negative control due to the absence of Cdx1 as an A33-inducing factor. Total RNA was isolated from all different segments and liver and was used for RT-PCR. It was found that the A33-CathB-EGFP mice did not express the transgene in any of the intestinal parts (Figure 2, upper panel) while CHO-K1 cells co-transfected with pCdx1-DsRed-Express and pA33-CathB-EGFP gave the expected band at 1606 bp. These data suggested that there was possibly a problem at the transcriptional level that resulted in a lack of expression of the transgene in the A33-CathB-EGFP mice. We then checked the expression of the endogenous mouse cathepsin B and no difference was observed between wild type and transgenic mice (Figure 2, middle panel), showing that integration of the A33-CathB-EGFP construct did not influence expression of the endogenous protease.

A33-CathB-EGFP mice do not translate the chimeric protein cathepsin B-EGFP in their intestine

Immunoblotting experiments with a GFP-specific antibody were performed in order to confirm the lack of cathepsin

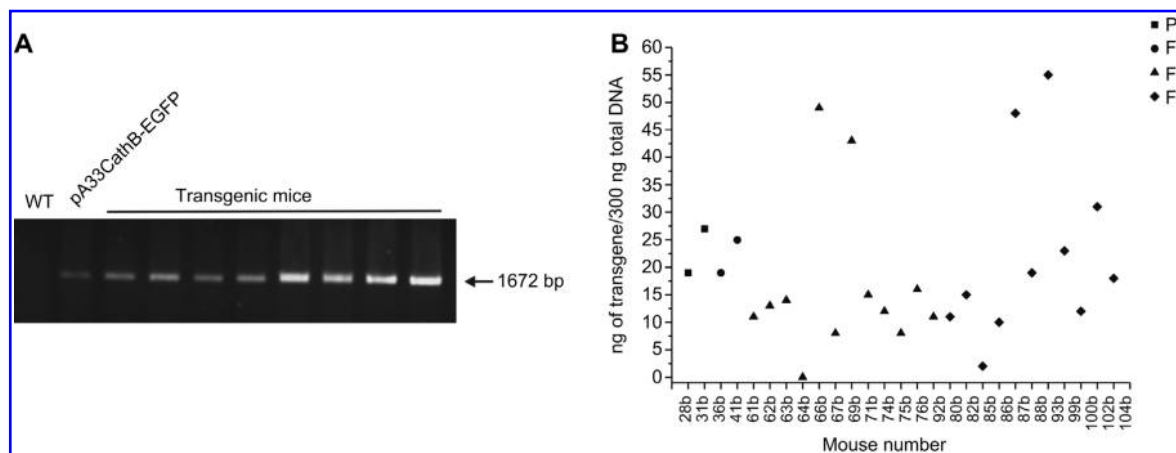


Figure 1 Genotyping of the A33-CathB-EGFP transgenic mice.

(A) Semi-quantitative PCR analysis using a transgene-specific primer pair shows different amounts of transgene in the various A33-CathB-EGFP transgenic mice. No PCR product was detected for wild type mouse (WT) while the plasmid pA33-CathB-EGFP served as positive control. (B) Scattergram of the analyzed A33-CathB-EGFP mice indicating the amount of transgene through three generations. Note that the transgene was kept and present in comparable copy numbers over the different generations.

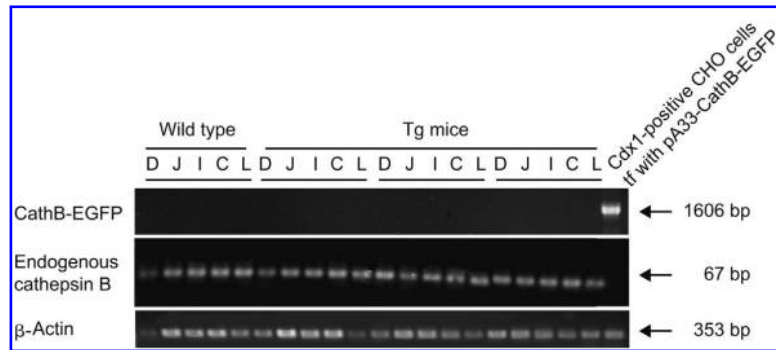


Figure 2 Expression of A33-CathB-EGFP transgene and endogenous cathepsin B.

Total RNA isolated from intestinal segments (D=duodenum, J=jejunum, I=ileum, C=colon) and liver (L) of wild type and transgenic mice was used for RT-PCR with two pairs of primers that can distinguish between chimeric rat cathepsin B-EGFP and endogenous mouse cathepsin B. CHO-K1 cells co-transfected with pCdx1-DsRed-Express and pA33-CathB-EGFP were used as positive control. By using the rat cathepsin B-specific primers, an RT-PCR product of 1606 bp was only detected for co-transfected CHO-K1 cells but not for the wild type or any of the transgenic mice. Mouse cathepsin B-specific primers gave the expected product of 67 bp in both wild type and transgenic mice but not in co-transfected CHO-K1 cells. The β -actin gene product was used as a control for RNA integrity.

B-EGFP also at the protein level. Intestine tissue extracts were prepared from wild type and transgenic mice, as well as cell lysates from non-transfected, single (pCdx1-DsRed-Express) or co-transfected (pCdx1-DsRed-Express and pA33-CathB-EGFP) CHO-K1 cells. The latter cell lysates served as positive controls of cathepsin B-EGFP expression under the control of the A33-antigen promoter. The band at approximately 60 kDa corresponding to the molecular mass of the chimeric protein was detected only in lysates of co-transfected CHO-K1 cells, thereby reproducing the results of our previous proof-of-principle experiments (Mayer et al., 2008). However, no signal corresponding to cathepsin B-EGFP was detected in the intestine tissue extracts prepared

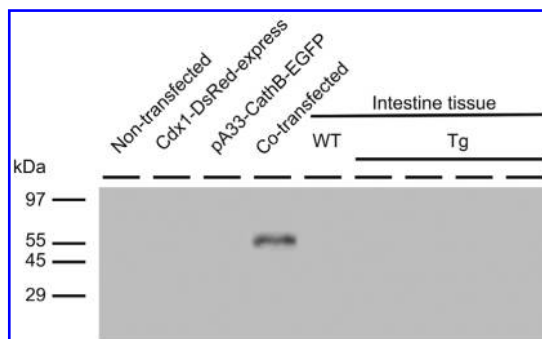


Figure 3 Transgenic mice do not express cathepsin B-EGFP chimeric protein.

Immunoblot analysis was performed using GFP-specific antibody on cell lysates and tissue extracts. Cell lysates were prepared from non-transfected, single or co-transfected CHO-K1 cells while intestine tissue from wild type and transgenic mice was used for preparation of tissue extracts. The band at approximately 60 kDa corresponding to the molecular mass of cathepsin B-EGFP chimeras was only detected in lysates of CHO-K1 cells co-transfected with pCdx1-DsRed-Express and pA33-CathB-EGFP plasmids, but not in non-transfected or singly transfected cells or wild type or transgenic mice. Molecular mass markers are given in the left margin.

from both wild type and transgenic mice (Figure 3). These results confirmed that the chimeric protein was not translated in the intestine of A33-CathB-EGFP mice even though its coding sequence was integrated into the mouse genome and kept over three generations at well detectable levels.

Cryosections of small and large intestine and also liver were prepared from wild type and transgenic mice and were checked for green fluorescence by microscopic analysis. Green fluorescence signal was detected in a few cells (Figure 4, arrows) but the intensity was equal in the tissue of both wild type (Figure 4, A–E) and transgenic mice (Figure 4, F–L), suggesting that such signals were due to tissue auto-fluorescence and not to the presence of the chimeric protein cathepsin B-EGFP. In an effort to enhance a potentially weak signal of cathepsin B-EGFP, an antibody specific for GFP was used for immunolabeling of intestine tissue cryosections of transgenic and wild type control animals. In this case we used a secondary antibody coupled with red-emitting fluorophore in order to avoid cross-talk with the signal from the chimeric protein. However, no fluorescence signal was observed in the intestinal tissue of transgenic mice stained with anti-GFP antibodies (Figure 5, F–L). The same result was also observed in the wild type animals (Figure 5, A–E). These studies therefore confirmed the lack of translation of cathepsin B-EGFP chimeric protein in our transgenic mouse model.

A33-CathB-EGFP and wild type mice revealed comparable intestinal morphology

Morphological studies were performed in an effort to investigate whether the A33-CathB-EGFP mice had a generally altered intestinal phenotype due to integration of the transgene. We first stained the nuclei with DRAQ5TM (Biostatus Limited, Shepshed, Leicestershire, UK) in order to be able to compare the cell numbers of the different intestinal parts and also of the liver between transgenic and wild type mice. By this experiment we expected to also observe potential alterations in the intestinal structure. We observed that the

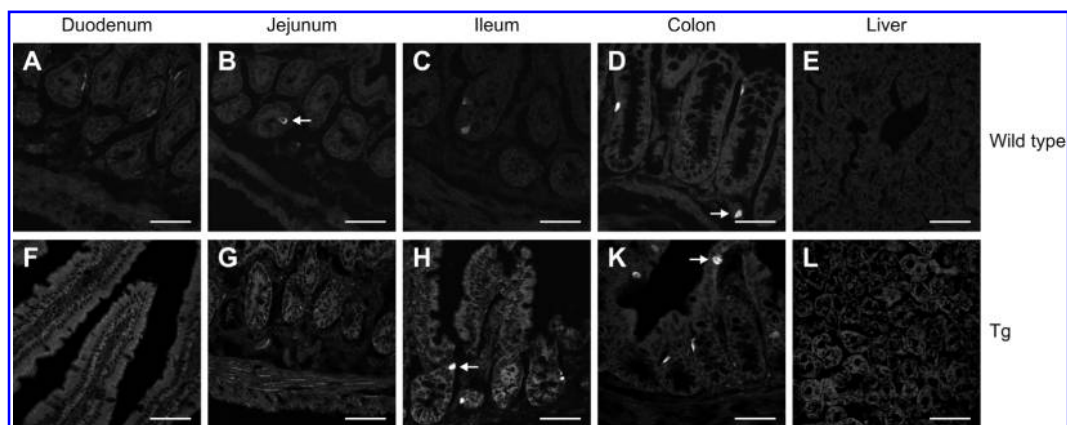


Figure 4 Lack of green fluorescence in the intestine of transgenic mice.

Cryosections of intestine segments and liver isolated from wild type (A–E) and transgenic mouse (F–L). Tissue cryosections were inspected by fluorescence microscopy under blue light excitation required to directly detect the introduced EGFP tag without any prior immunolabeling. Liver served as negative control. Green fluorescence signals were detected in a few cells (arrows) but these were of equal intensities for both wild type and transgenic mice, indicating that such fluorescence signals were due to tissue auto-fluorescence. Bars, 50 μ m.

cell numbers found in the intestine and liver of transgenic mice were comparable to those found in the control animals (Figure 6). Moreover, the structure of the intestine did not show any alterations in the A33-CathB-EGFP mice, implying that integration of the transgene did not result in any developmental changes during tissue morphogenesis (Figure 6, phase contrast pictures). In addition, an antibody that can detect both the endogenous (mouse) and the chimeric (rat) cathepsin B was used in order to check whether the localization and expression pattern of the endogenous protein was altered in the transgenic mice. However, the amounts and localization of endogenous cathepsin B were comparable between wild type (Figure 7, A–E) and transgenic (Figure 7, F–L) animals.

The expression pattern and localization of the brush border enzyme aminopeptidase N (APN) was also analyzed. APN is an integral plasma membrane protease with a broad sub-

strate specificity that serves as an apical plasma membrane marker in the small intestine (Mina-Osorio, 2008). The reason we wanted to study such markers was to check whether the polarity of the intestinal epithelium was affected in the transgenic mouse model. Cryosections of intestine and liver tissue were used for immunofluorescence analysis and confocal laser scanning micrographs prepared from transgenic and wild type mice revealed a steady expression of APN (green color) without changes in the distribution of this protein in both transgenic (Figure 8, F–L) and wild type (Figure 8, A–E) mice. Note that localization of APN at the apical plasma membrane in the small intestine (Figure 8, A–C and F–H) is replaced by basolateral plasma membrane localization in the large intestine (Figure 8, D and K). The results from both the small and large intestine suggested that the overall structure and polarity of this tissue was not affected in A33-CathB-EGFP mice.

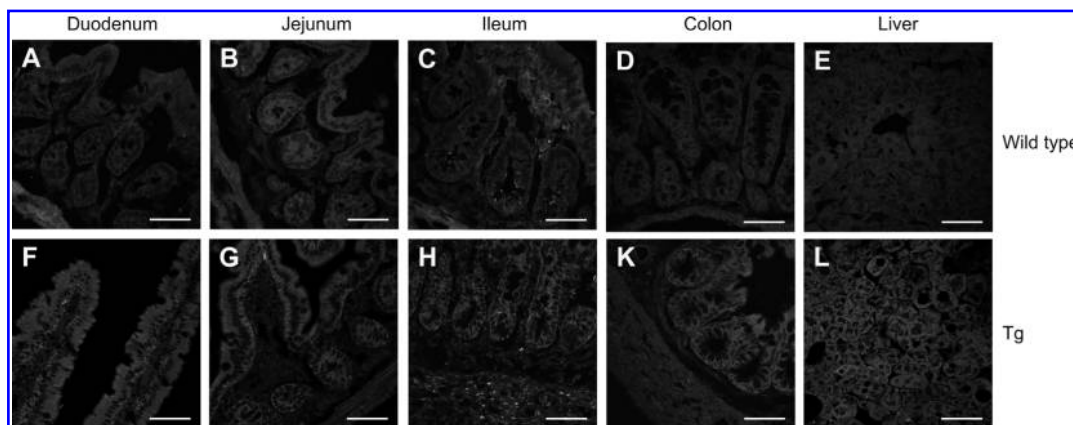


Figure 5 Enhancement of GFP signal by immunolabeling.

In order to enhance a potentially weak green fluorescence emitted by CathB-EGFP chimeras, tissue cryosections from wild type (A–E) and transgenic mice (F–L) were immunolabeled with GFP-specific and red fluorophore-conjugated secondary antibodies. No specific red fluorescence was detected, again confirming lack of cathepsin B-EGFP protein. Bars, 50 μ m.

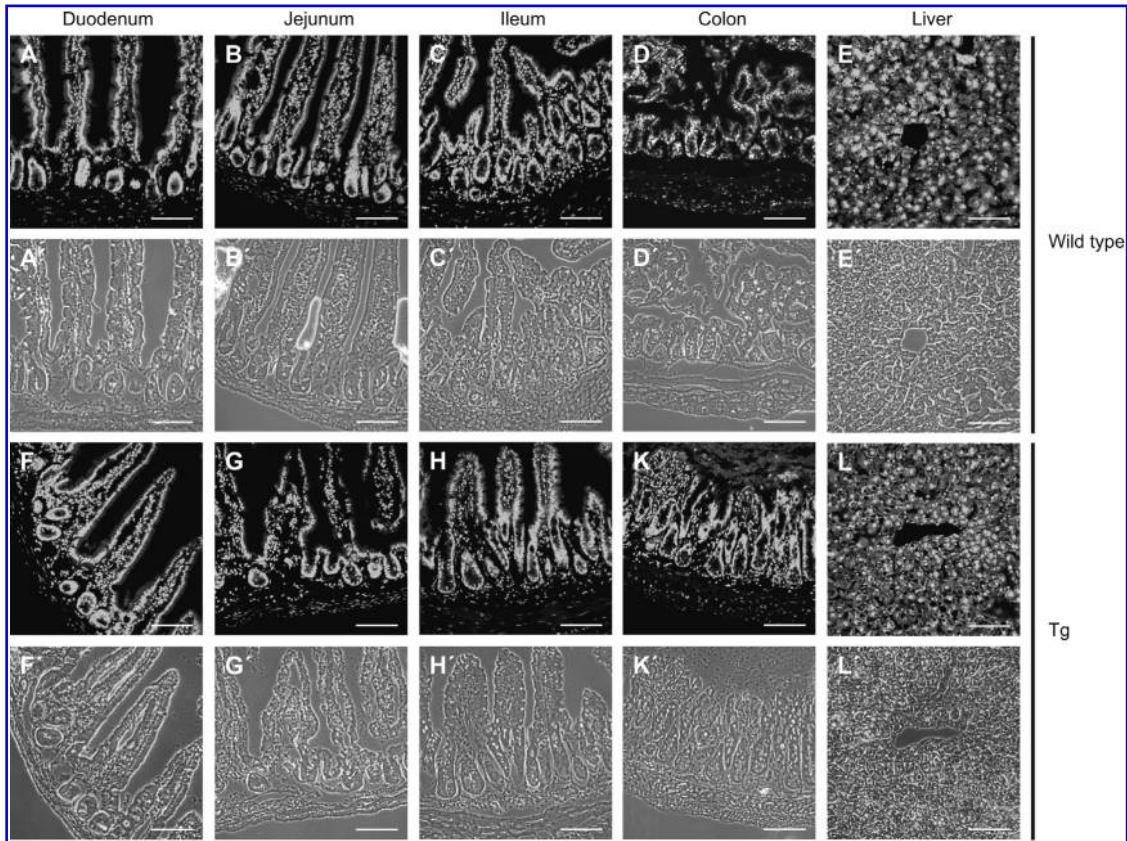


Figure 6 Comparison of intestinal morphology by nuclei staining.

The cell numbers of the different intestinal segments and the liver were analyzed by fluorescence microscopy after staining with the nuclear marker DRAQ5TM. Corresponding phase contrast micrographs are also shown. No morphological alterations of intestine or liver tissue were observed in the transgenic animals (F–L) when compared to the wild type controls (A–E). Bars, 50 μ m.

Expression and localization of Cdx1 and A33 antigen in the intestine of A33-CathB-EGFP mice

In an attempt to identify reasons for the non-expression of cathepsin B-EGFP chimeric protein in the intestine of

A33-CathB-EGFP mice, we next wanted to check whether a general problem with the regulation of the A33-antigen promoter occurred upon transgene incorporation. For this purpose, we focused our studies on the intestine-specific transcription factor Cdx1 that is crucial for A33-antigen pro-

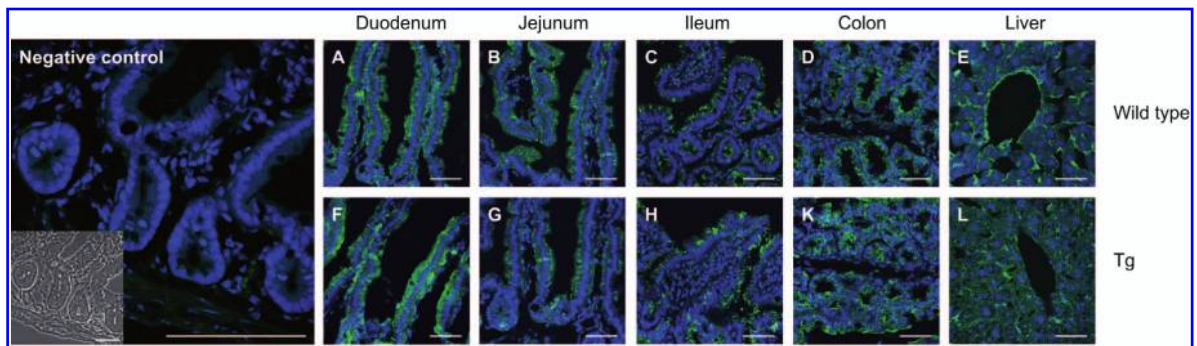


Figure 7 Cathepsin B follows the same expression and localization pattern in wild type and transgenic mice.

Immunofluorescence analysis was performed using a polyclonal cathepsin B-specific antibody that recognizes both the endogenous (mouse) and the chimeric (rat) protein. The expression levels as well as the localization of endogenous cathepsin B (green fluorescence) remained unaltered in the transgenic mice in comparison to wild type animals, confirming normal intestinal morphology of those animals. DRAQ5TM was used as nuclear counter-stain (blue fluorescence). Negative controls are shown on the left. Bars, 50 μ m.

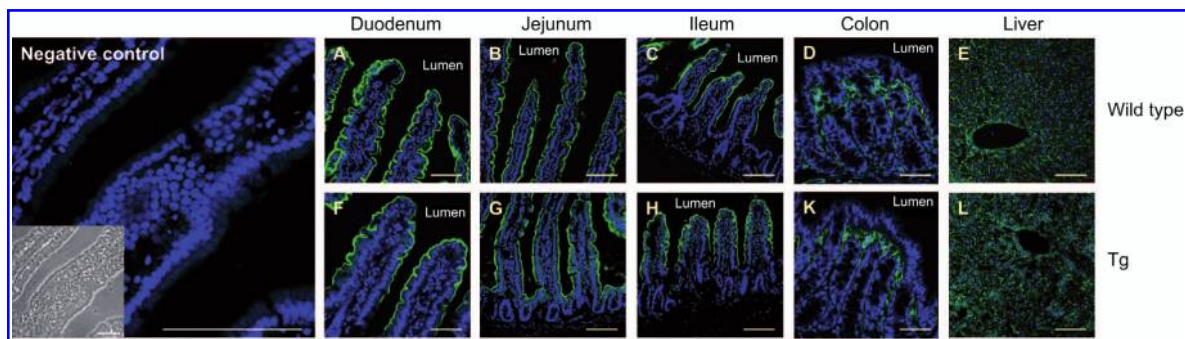


Figure 8 Expression and localization of the brush border enzyme aminopeptidase N.

Confocal laser scanning micrographs of cryosections prepared from wild type (A–E) and transgenic (F–L) mice after staining with APN-specific antibody. A steady expression of APN (green fluorescence) without changes in the localization of this enzyme was observed in both wild type (A–E) and transgenic (F–L) mice. Note the localization of APN at the apical plasma membrane in the small intestine (A–C and F–H) that is replaced by basolateral plasma membrane localization in the colon (D and K). DRAQ5™ was used as nuclear counterstain (blue fluorescence). Negative controls are shown on the left. Bars, 50 μm .

motor activation, and we also investigated distribution of the intestinal protein A33 antigen which is under the control of this same promoter. Cryosections of intestine tissue isolated from transgenic mice were used for these studies in which the presence of Cdx1 (Figure 9A and B) was confirmed in the intestine, but not in tissues such as heart and liver which are known to be Cdx1-negative in mice (Figure 9C and D). Cdx1 protein was present in differentiated cells of the intestinal epithelium only, but absent from the stem cells at the

crypt base (Figure 9B), as expected. Furthermore, the Cdx1-response gene A33 antigen was expressed as obvious from the presence of A33 antigen on the protein level in the intestine of the transgenic mice (Figure 9E). Both Cdx1 and A33 antigen were localized as expected in the nuclei of enterocytes and at the basolateral plasma membrane of intestinal epithelial cells, respectively. These observations ruled out the possibility that lack of transgenic cathepsin B-EGFP expression in the intestine of A33-CathB-EGFP mice was due to

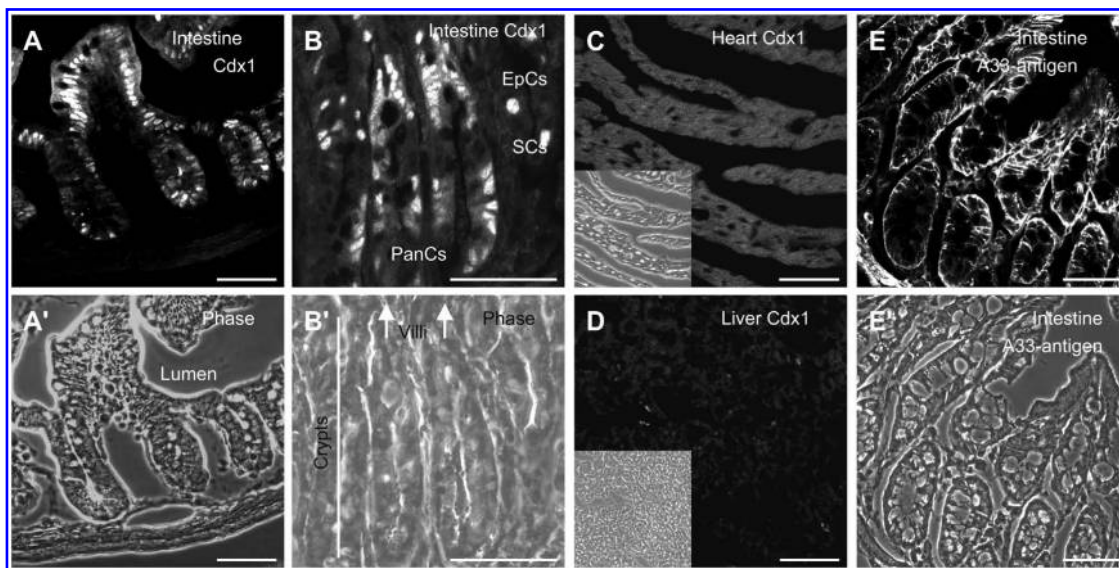


Figure 9 Presence and localization of Cdx1 and A33 antigen in the intestine of A33-CathB-EGFP mice.

Confocal laser scanning micrographs of cryosections prepared from intestine (A, B, E), heart (C) and liver (D) tissue of A33-CathB-EGFP mice after staining with Cdx1 and A33 antigen-specific antibodies as indicated. Cdx1 was observed within the nuclei of enterocytes (A and B) whereas the A33 antigen was found in the basolateral plasma membrane domain of intestinal epithelial cells (E). Corresponding phase contrast micrographs are also shown (A'–E'). Higher magnification of the crypt regions (B) revealed absence of Cdx1 in stem cells at position +4 but its presence in differentiated cells of the mouse intestinal mucosa as expected. Cdx1 was absent from heart and liver tissue (C–D). The presence of Cdx1 and A33 antigen in the transgenic mouse intestine and their localization patterns following the expected distribution reject the possibility of altered and non-functional regulation in general of the intestine-specific Cdx1-driven expression of genes under the control of the A33-antigen promoter. EpCs, epithelial cells; SCs, stem cells and PanCs, Paneth cells. Bars, 50 μm .

altered regulation of the A33-antigen promoter, or was caused by alterations in the differential expression pattern of Cdx1, its inducing and intestine-specific transcription factor.

Discussion

Cysteine cathepsin-deficient and transgenic mouse models for analysis of proteolytic functions of this diverse family of proteolytic enzymes

Cathepsin-deficient mice have been important tools in elucidating the roles and functions of these proteases *in vivo* (Reiser et al., 2010). Among them, cathepsin B-deficient animals exhibit a normal phenotype and cannot be distinguished from wild type littermates unless they are challenged (Guicciardi et al., 2000; Halangk et al., 2000; Reinheckel et al., 2001; Friedrichs et al., 2003; Brix et al., 2008). In this study we wanted to create a transgenic mouse model that would enable us to investigate the physiological role and trafficking of cathepsin B *in vivo*. The A33-CathB-EGFP mice were generated based on a principle previously proved functional in an *in vitro* cell culture model (Mayer et al., 2008) for expression of the chimeric protein cathepsin B-EGFP under the A33-antigen promoter that was induced by co-expression of the intestine-specific transcription factor Cdx1. Furthermore, it was initially envisaged that, apart from being able to monitor and visualize cathepsin B *in situ*, the transgenic mouse model would also serve as a system to analyze transgenic over-expression of cathepsin B. As such the A33-CathB-EGFP mice would be ideal for comparison with cathepsin B-deficient mice, since they would be two opposite cases, one of cathepsin B over-expression and one of cathepsin B absence.

Moreover, the reason we were interested in investigating the function of cathepsin B specifically in the intestine was previous studies of our group suggesting a role of cathepsin B in the ECM remodeling of mouse intestine (Vreemann et al., 2009). More specifically, it was suggested that cathepsin B might have a role in collagen IV degradation. Collagenase activity has also been shown for other cathepsins, for example cathepsin K, which is known to be involved in bone remodeling (Saftig et al., 1998; Li et al., 2002). Recently, we have shown that cathepsin K deficiency affects the gastrointestinal tract structurally (Dauth et al., 2011). Therefore a comparison of the intestinal tasks of cathepsin B that resides within endo-lysosomes of human enterocytes versus cathepsin K that is secreted from intestinal cells (Mayer et al., 2006) would have been interesting and within reach. Another aspect of interest would be to elucidate potential alterations in the overall intestinal tissue architecture and function due to over-expression of cathepsin B. It is well established that misbalance of expression or localization of proteases and their inhibitors is often associated with pathological conditions of the gastrointestinal tract, such as inflammation of the intestine (Medina and Radomski, 2006). In a mouse model of colitis, for example, it was shown that inhibition of cathepsins B and L or of cathepsin D can result in reduced damage of mucosal tissue (Menzel et al., 2006). Hence,

cysteine cathepsins may not simply be considered endo-lysosomal proteolytic enzymes important for general protein turnover, but they may entail more specific functions in gastrointestinal tissues.

Transgenic mice develop normally

The first step in characterizing the generated A33-CathB-EGFP mice was to confirm incorporation of the transgene. We found that the transgene was successfully integrated and was kept over three generations while the A33-CathB-EGFP mice had no obvious phenotype and showed normal breeding behavior. For our further studies, we focused on each intestinal segment (duodenum, jejunum, ileum, and colon) separately since these parts are known to have unique functional and morphological features. Moreover, the expression pattern of various intestinal proteins is graded along the anterior-posterior axis, as in the case of the intestine-specific transcription factor Cdx1, for which the highest expression levels are observed in the distal colon (Duluc et al., 1997). Thus, we investigated the different parts of the intestine for expression of the A33-CathB-EGFP transgene, because the highest protein levels could have been predicted to be expressed in the colon due to the high amounts of Cdx1. However, no expression of the chimeric protein was observed in any of the intestinal segments of the A33-CathB-EGFP transgenic mice. On the other hand, expression of the endogenous mouse cathepsin B was detectable in all intestinal parts, and reassuringly without any differences observed between wild type and A33-CathB-EGFP mice.

Based on these results we continued our studies at the protein level. Immunoblotting experiments showed lack of the chimeric protein cathepsin B-EGFP in the intestine of A33-CathB-EGFP mice, a finding that was also confirmed by morphological studies in which no green fluorescence was observed.

The above results then led us to a more general question of whether the A33-CathB-EGFP mice had an overall altered intestinal phenotype due to transgene integration. Could it be that the A33-CathB-EGFP transgene was incorporated in a site leading to expression differences of other proteins associated with tissue structure, polarity and function? To answer this question we performed morphological studies in which the cell numbers of the different intestinal parts were compared between wild type and A33-CathB-EGFP mice as well as their general structure. Since no alterations were observed, we suggest that integration of the transgene had no effect during development and tissue morphogenesis. Furthermore, immunostainings for cathepsin B confirmed our *in vitro* studies (Mayer et al., 2008) where we had shown that the localization and trafficking of the endogenous cathepsin B is not altered due to the presence of the chimeric CathB-EGFP.

An important function of the gastrointestinal tract is to serve as a barrier, protecting the organism from the various pathogens found in the intestinal lumen (Schneeman, 2002; Turner and Turner, 2010). This barrier function strongly depends on the polarity of the intestinal epithelium, which is why we wanted to check for potential changes in such polarity-associated markers. One of those markers is the

APN also known as CD13. APN is a brush border enzyme that serves as an apical plasma membrane marker in the small intestine but in the large intestine it is found in the basolateral plasma membrane domain. APN, also called moonlighting enzyme, is of major interest since multiple functions, including differentiation, proliferation, apoptosis and signal transduction, have been assigned to this protein (Mina-Osorio, 2008). The localization and expression pattern of APN was not changed in the A33-CathB-EGFP mice, another finding suggesting that these mice were comparable to wild type littermates and that transgene integration was not compromising homeostasis of the intestine.

Conclusions

Since our analyses showed that the transgenic mouse model generated in this study had no dramatic alterations in the overall intestinal phenotype and that the animals were similar to wild type mice, the key question remaining is why we were not able to detect cathepsin B-EGFP?

In order to approach this question in more detail we wanted to check whether a potentially altered regulation of the A33-antigen promoter had led to a lack of cathepsin B-EGFP translation in the intestine of transgenic mice. For instance, a potential absence of Cdx1 could have resulted in non-expression of the chimeric protein because this intestine-specific transcription factor is known to be crucial for the activation of the A33-antigen promoter (Johnstone et al., 2002). Moreover, the levels of Cdx1 would also affect the extent of expression of one of its target genes, the A33 antigen in the mouse intestine. However, by immunofluorescence analysis we confirmed the presence of both Cdx1 and A33 antigen, as well as their expected localization patterns in the intestine of A33-CathB-EGFP animals. These results strongly suggested that Cdx1-driven gene expression and translation of proteins under the control of the A33-antigen promoter were still functional in the transgenic animals that were generated in this study.

We concluded that problems in the regulation and activation of the A33-antigen promoter were not the reason for the observed lack of cathepsin B-EGFP. On the other hand, a possible reason for this would be that the transgene was integrated in a site where it was silenced, resulting in no transcription. Another possibility could be the production of unstable mRNA, which we were not able to detect due to rapid degradation. One crucial factor for RNA degradation is the truncation of the polyA-tails of transcription products (Couttet et al., 1997; Brown and Johnson, 2001). The pA33-CathB-EGFP plasmid was linearized prior to its microinjection into the pronuclei of developing zygotes, meaning that, indeed, the polyA-tail might have been shortened or eliminated during the process. Another likely explanation for the lack of chimeric protein translation is the absence of an internal sequence that would promote splicing during transcript generation, and hence premature disintegration of transcripts was possibly involved (Choi et al., 1991; Palmiter et al., 1991; Auerbach, 2004). In another recent study in which the A33-antigen promoter was used in order to express human *TGF-βRII* specifically in the intestine of transgenic mice

[Flentjar et al., 2007], such splicing was planned for and transgenic expression in an intestine-specific manner was achieved. We therefore conclude that expression of soluble cysteine cathepsins under the control of a promoter that drives intestine-specific expression of a typical enterocyte transmembrane protein was not productive.

Further experiments are planned to better understand the regulation of cysteine cathepsin expression in intestinal tissues. So far, it is known that immunosuppressive transforming growth factor- β 1 (TGF- β 1) results in down-regulation of cathepsin B and L expression, whereas the proinflammatory cytokine IL-6 leads to dose-dependent up-regulation of cysteine cathepsin expression (Gerber et al., 2001; Reisenauer et al., 2007). Similarly, IL-13 is known to stimulate the expression of matrix metalloproteinases but also of cathepsins in the lungs of mice suffering from emphysema (Zheng et al., 2000). Furthermore, activation of specific, pro-inflammatory caspases was proposed to be modulated by cathepsin-mediated proteolysis (Schotte et al., 1998). It is therefore a possible hypothesis (but beyond the scope of this report) that in addition to Cdx1, further factors, normally accounting for an efficient immune response during acute or chronic inflammation, would have been required to regulate the expression levels of cysteine peptidases such as cathepsin B in the transgenic mouse model realized in this study. Our own results with a trauma model of surgical manipulation of the mouse intestine argue in favor of this latter proposal, because only transient and localized inflammatory responses were observed after surgery while cathepsin B mRNA levels remained constant throughout the regeneration phase of several days (Vreemann et al., 2009). The localization pattern of cathepsin B was, however, dramatically changed within a few hours, both in our *in vitro* (Mayer et al., 2009) and in the *in vivo* model of intestinal manipulation (Vreemann et al., 2009). The findings of these and of the present study may thus indicate that a redundant system of cysteine peptidases in the gastrointestinal tract ensures rapid reactions to challenging conditions by protease re-location rather than by alteration in the protease expression levels.

Materials and methods

Generation of A33-CathB-EGFP transgenic mice

In order to establish a transgenic mouse model, pA33-CathB-EGFP (Mayer et al., 2008) was linearized by digestion of 100 μ g plasmid DNA with ApaLI in Y⁺ Tango buffer (both from MBI Fermentas, St. Leon-Rot, Germany) for 1 h at 37°C. Inactivation of the restriction enzyme was performed for 20 min at 65°C, the linearized plasmid DNA was purified using a PCR Purification Kit (Qiagen, Hilden, Germany) and used for microinjection into the pronuclei of developing zygotes. Pronuclear injection into fertilized C57BL/6NcrI oocytes was performed by T.G. and R.S. at the Transgenic Core Facility of the Freiburg University Medical Center (Freiburg, Germany), yielding the founder transgenic mice, A33-CathB-EGFP, which were transferred to the Jacobs University Bremen. Housing and breeding of wild type and transgenic animals were conducted in accordance with institutional guidelines and took place in the S1-

laboratories of Jacobs University Bremen, Germany, registered as SfAFGJS Az. 513-30-00/2-15-32 and 522-27-11/3-1, 05-A20 and A21. Mice were housed under standard conditions, with a 12 h/12 h light/dark cycle with lights on from 07:00 to 19:00, and *ad libitum* water and food.

Genotyping of the A33-CathB-EGFP transgenic mice

Tail biopsies of the transgenic animals were analyzed in order to examine the incorporation of the transgene by using semi-quantitative PCR. Total DNA was isolated with DNeasy Blood and Tissue Kit (Qiagen) and 300 ng were used as a template for PCR reactions. The primers used for genotyping were designed based on the published sequence of pEGFP-N1 (Clontech Laboratories, Heidelberg, Germany). The forward primer (5'-GTT ATC CCC TGA TTC TGT GG-3') is located upstream of the *Asel* restriction site and the reverse primer (5'-GTG GCG ACC GGT GGA TC-3') binds downstream of the *Bam*HI restriction site that was used for generation of pCathB-EGFP (Linke et al., 2002; Mayer et al., 2008). PCR products were separated on 1% agarose gels and visualized by inclusion of 0.3% ethidium bromide.

Tissue preparation

A33-CathB-EGFP and wild type mice were anesthetized, the abdominal and thoracic cavities were opened and the abdominal aorta was cut. For perfusion via the heart, 0.9% NaCl supplemented with 200 IU heparin (Braun Melsungen AG, Melsungen, Germany) was used. Subsequently, the small and large intestines were isolated and kept on ice. The mesenteries were removed and the intestines were washed with ice-cold 0.9% NaCl solution. The small intestine was further divided into its three segments, duodenum, jejunum, and ileum. Apart from the small and large intestine (colon), the heart and liver were also obtained serving as negative controls. Each intestinal segment was divided into two parts. The anterior part was fixed using 4% paraformaldehyde (PFA) in 200 mM 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid (HEPES), pH 7.4, and used for morphological studies while the posterior part was snap-frozen in liquid nitrogen and used for biochemical analysis.

Analysis of transgene expression

Total RNA was isolated from the different intestinal segments and the liver using the RNeasy Mini Extraction Kit and subjected to DNase treatment according to the manufacturer's instructions (Qiagen). Reverse transcription was performed with the Omniscript Reverse Transcription Kit (Qiagen) at 37°C for 1 h. Each reaction contained 2 µg RNA, 0.5 mM dNTPs, 1 µM random oligonucleotide primers, and 4 U Omniscript Reverse Transcriptase. The cDNAs were amplified with the following primers: 5'-CCT TGA TCC CTC TCT CTT GCC TGC-3' (forward) and 5'-TGG TTG TCG GGC AGC AGC AC-3' (reverse) to include the coding regions of the entire chimera consisting of cathepsin B and EGFP. With this primer combination the endogenous mouse cathepsin B is not amplified. PCR reactions were performed in a total volume of 20 µl in the presence of 2 µl RT reaction buffer, 100 pmol of each specific primer, 2 U *Taq* DNA polymerase, 1.25 mM MgCl₂ and 0.2 mM dNTPs (MBI Fermentas). In addition, primers amplifying the endogenous mouse cathepsin B were used (forward, 5'-TGC GTT CGG TGA GGA CAT AG-3' and reverse, 5'-CGG GCA GTT GGA CCA TTG-3'), as well as primers specific for β-actin (forward, 5'-GCT CGT CGT CGA CAA CGG CTC-3' and reverse, 5'-CAA ACA TGA TCT GGG TCA TCT TCT C-3').

Immunoblot analysis

Total tissue extracts were isolated with lysis buffer PBS containing 0.5% Triton X-100, and homogenization of the samples was done using a Potter S homogenizer (Sartorius, Göttingen, Germany) at 1000 rpm for 5 min on ice. Homogenates were kept in a rotary mixer for 45 min while all steps were performed at 4°C. After centrifugation for 10 min at 10 000 g, supernatants were stored at -20°C. Protein concentration was determined using BSA as a protein standard (Neuhoff et al., 1979). For each sample, 10 µg of tissue extract was boiled in sample buffer consisting of 10 mM Tris-HCl, pH 7.6, 0.5% (w/v) SDS, 25 mM DTT, 10% (w/v) glycerol, and 25 µg/ml bromophenol blue. Prestained protein standards were used as molecular mass markers (MBI Fermentas). Samples were analyzed on 8–12.5% gradient acrylamide gels and then blotted onto nitrocellulose membranes using a semi-dry blotting procedure. After blocking overnight at 4°C with 5% milk powder in PBS containing 0.3% Tween-20, primary antibodies were applied. The antibodies used were goat anti-mouse cathepsin B (Neuromics, through Acris Antibodies, Herford, Germany), mouse anti-GFP (Roche Diagnostics, Mannheim, Germany), and rabbit anti-mouse A33 antigen (Abcam, Cambridge, UK). After several washing steps, membranes were incubated for 1 h at room temperature with HRP-conjugated rabbit anti-goat, goat anti-mouse, and goat anti-rabbit secondary antibodies (all from Southern Biotech, Birmingham, Alabama, USA). Visualization of immunoreactions was achieved by using enhanced chemiluminescence substrate on CL-XPosure film (both from Pierce through Perbio Science Europe, Bonn, Germany).

Morphological analysis by immunolabeling

The three small intestine segments, the colon, and the liver were fixed in 4% PFA in 200 mM HEPES buffer, pH 7.4 and left overnight at 4°C. PFA was washed out and samples were incubated overnight in 200 mM HEPES buffer (pH 7.4). Intestine samples were then cut into pieces of approximately 1 cm length and segments representing duodenum, jejunum, and ileum were taken from the beginning, middle, and end parts, respectively. The above pieces were incubated overnight in Tissue Freezing Medium (Jung, through Leica Microsystems, Nussloch, Germany), and were subsequently frozen on dry ice.

Cryo-sections of 5 µm were prepared from each sample by using a Leica CM1900 cryostat (Leica Microsystems) and mounted on microscope slides. Prior to staining, slides were incubated with PBS overnight at 4°C in order to remove the remaining embedding material. Non-specific binding sites on sections were blocked with 3% bovine serum albumin (BSA) which was followed by incubation with primary antibodies diluted in 0.1% BSA in calcium- and magnesium-free (CMF) PBS.

Specific primary antibodies were rabbit anti-GFP (Abcam), goat anti-mouse cathepsin B (Neuromics), rabbit anti-human Cdx1 (Abcam), rabbit anti-mouse A33 antigen (Abcam), and goat anti-mouse aminopeptidase N (R&D Systems, Wiesbaden, Germany). After washing, sections were incubated for 1 h at 37°C with secondary Alexa-543-coupled goat anti-rabbit and rabbit anti-goat IgG (Invitrogen through Molecular Probes, Karlsruhe, Germany). DRAQ5TM (Biostatus Limited, Shepshed, Leicestershire, UK) in a final concentration of 5 mM served as a nuclear counter-stain. Negative controls were prepared in which the specific primary antibodies were omitted and sections were incubated only with secondary antibodies and DRAQ5TM. Immunolabeled samples were viewed with a Zeiss LSM 510 META laser scanning microscope equipped with Argon and Helium-Neon lasers (Carl Zeiss GmbH, Jena, Germany). Optical sections were obtained with a pinhole

setting of 1 Airy unit and at a resolution of 1024×1024 pixels and were further analyzed by using LSM 510 software, Release 3.2 (Carl Zeiss).

Cell lines and transfections

CHO-K1 cells were used for transfection with pCdx1-DsRed-Express and pA33-CathB-EGFP vectors in order to serve as positive control for RT-PCR reactions and for immunoblotting. Cell culture and transfection procedures were performed as previously described (Mayer et al., 2008).

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